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Production and Unsaturation Index of Alkenones During Batch and Continuous Cultures of the Coccolithophorid Alga, *Emiliania Huxleyi*

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Abstract

Emiliania huxleyi (EH2 strain) was grown at 10, 15, 20 and 250C in batch and continuous cultures to assess the factors affecting the changes in the production and the unsaturation index of alkenones. The production of alkenones continued during growth and was greatly stimulated at IOOC where cell growth was greatly suppressed, but not influenced by the changing cell size. Alkenones are chemically and/or biologically stable compound for they can be detected even in broken or dead cells. The UK_{37} and the ratio between $C_{36:2-}$ ethyl alkenoate (EE) and total C_{37} alkenones (K_{37}) (EE/K_{37}) changed during growing phase and remained nearly constant during the stationary phase at all temperatures tested in batch cultures. Data in continuous culture showed that the alkenones with 2 double bonds increased at high temperature. While the alkenones with 3 double bonds decreased and the reverse change was clearly observed when temperature was decreased. $U^{K'}{}_{37}$ changed without any lag when temperature changed and needed approximately 2-6 days to attain the respective levels depending on the difference of temperature given. The final values of $U^{K'}{}_{37}$ obtained at stationary stage were similar between batch and continuous cultures at each temperature and the values increased with increasing temperature. The results strengthened the suggestion that temperature is the major factor that influences the production and unsaturation of alkenones.

Keywords: alkenones, $U^{K'}{}_{37}$, coccolithophorids, batch culture, continuous culture, organic compounds

Introduction

The cosmopolitan micro-alga, *Emiliania huxleyi* (Hyptophyta: Haptophyceae), has frequently been reported to form large blooms and to be the carbon cycle on a global scale. Under illumination, they fix inorganic carbon by both photosynthesis and calcification. However, calcification involves the release of carbon dioxide i. e. $Ca^{2+} + 2HCO_3$, $CaCO_3 + CO_2 + H_2O$ (Brand, 1982; Westbroek et al., 1993; Harris, 1996).

Alkenones are organic compounds of long-chain unsaturated methyl and ethyl ketones that were discovered 20 years ago (Boon et al., 1978; de Leeuw et al., 1980; Volkman et al., 1980). These compounds contain a carbonyl group and di-, tri- and tetra-unsaturations with a rare trans configuration of double bonds in a C_{37} to C_{39} compound. They have been found throughout the world's oceans, except in the Arctic Ocean, both in its water column and sediment. The coccolithophorid, *E. huxleyi*, is the recognized predominant source of the long-chain alkenones that has been observed in marine sediment. The number of alkenones' double bonds is known to vary depending on the growth temperature at the time when they are synthesized in the cells. About 90% of alkenones are degraded during transport to the sediment but the degree of unsaturation during the time when they are synthesized at the sea surface are not affected by diagenetic process. Hence, the unsaturation index of alkenones ($U^{K'}_{37}$) has been widely employed in the late Quaternary period as biomarkers in estimating the paleo sea surface temperature (SST) (Prahl et al,v 1989; Brassell, 1993; Müller, et al., 1998). $U^{K'}_{37}$ was calculated by dividing the concentration of alkenones containing 37 carbons with 2 double bonds by the total concentration of C_{37} alkenones containing 2 and 3 double bonds (Brassell et al., 1986; prahl et al., 1988).

A linear $U^{K'}_{37}$ -temperature relationship has been established in batch culture experiments with E. huxleyi (Prahl and Wakeham, 1987; Prahl etal., 1988). However, the calibration lines presented in these studies differ from the studies of Volkman et al. (1995), Sawada et al. (1996), and Conte et al. (1998). These suggest the existence of some unknown factors affecting apart from the temperature. The localization and metabolic pathway for the synthesis of alkenones are not yet known.

The mechanism on the change in the degree of unsaturation in alkenone molecules is also unknown. Considering its importance in reconstructing paleotemperature: there is a need to establish a comprehensive knowledge about the various factors other than temperature, that may possibly influence the value of UK' and the mechanism for production and degradation. The present study was intended to show how the production of alkenone molecules and $U^{K'}_{37}$ are regulated during growth in batch and in continuous cultures of *E. huxleyi*. Alkenone studies with a chemostat culture are rare, and the data obtained here should provide new insight, particularly because they contradict the previous report of Popp et al. (1998). Details of previous study on the relationship between the age of the strain and the unsaturation and production of alkenones have not been found. It is believed that expected results are critical for alkenone studies and will be important for studying also the physiological functions of alkenones in Gephyrocapsean coccolithophorids.

Culture experiments

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The Emiliania huxleyi (Lohman) Hay & Mohler, strain EH2 that was isolated in 1990 from the Great Barrier Reef, SW Pacific, Australia was used as the algal material. The strain had been maintained for 11 years in 100-ml Erlenmeyer flasks containing 50 ml of an ESM-natural seawater medium under a 16-h light and 8-h dark cycle with 30 μmol m^{-2} s^{-1} irradiance at 20^0C as described by Sekino and Shiraiwa (1994). This strain was the same as those used by Sawada et al. (1996).

Pre-experimental cultures used cells harvested from the stock culture at the late logarithmic phase which were usually hoculated into a 500-ml Sakaguchi flask containing 300 ml of Marine Art SF artificial seawater (Senju Pharmaceutical Co., Osaka, Japan) with modified ESM enrichment, by which the soil extract was replaced with 10 nM selenite (Danbara and Shiraiwa, 199g). The cultures were maintained at various temperatures (10^0C 15^0C, 20^0C and 25^0C) under continuous illumination at an intensity of 35 μmol m^{-2} s^{-1} and gently hand-shaken once a day to re-suspend the sedimented cells. After 8 days, the cells were transferred to experimental Sakaguchi flasks.

The cell number was determined by microscopic counting with an MC-31 video monitor (Scalar, Tokyo, Japan), the optical density at 750 nm (OD_{750}) by a UV-VIS recording spectrophotometer (UV-2200, Shimadzu, Kyoto, Japan). The packed cell volume (PCV) was calculated from a calibration curve of OD_{750} versus PCV determined in a previous experiment The cells were harvested by filtration through a Whatman GF/F filter precombusted at 400"C for 3 h, and stored frozen at -200C until being used for extraction and analysis.

A continuous culture of the same strain was carried out at various temperatures in a photobioreactor (Able, Tokyo, Japan). Cells were grown in a pre-culture at 20^0C with air bubbling at a flow rate of 260 ml min^{-1} under continuous illumination of 30 μmol m^{-2} s^{-1} in a 500 ml oblong glass vessel, then inoculated into the bioreactor and allowed to grow at the desired cell density. Constant cell density was maintained in the culture by automated dilution with the same fresh medium as that used in the batch culture, The dilution rate was automatically controlled according to the algal growth rate by changing the amount of fresh medium input through a peristaltic pump connected to a turbidometer. The reaction vessel was a Pyrex glass cylinder of 150 mm i.d. x 300 mm height with a sufficient volume for 4 Of a medium: this being surrounded by twelve 10 W fluorescent lamps. The light intensity determined at the center of the vessel was 70 μmol m^{-2} s^{-1}. pH of 8.2 was automatically maintained by adding 0.1 N HCI or 0.1 N NaOH. The desired constant temperature was maintained by using a heater or by controlling the flow of cold water, as necessary. The culture was agitated at 50 rpm by a magnetic stirrer, bubbled with air at a flow rate of 0.4 1 min-I under continuous illumination and continuously monitored and controlled by a computer. ell data being recorded. At appropriate intervals, 20 ml and 150 ml aliquots of the cell suspension were harvested from the culture to determine the cell number under a microscope, the optical density and chlorophylls, and for the analysis or alkenones, respectively.

Analytical procedures

Lipids were extracted by four, 5-min rounds of sonication with 6 ml each of dichloromethane-methanol (6:4) and concentrated by rotary evaporation until the volume of the solvent was reduced to less than 0.5ml_ Samples were passed through a short bed of Na_2SO_4, (precombusted at 400^0C for 3 h) with glass wool to remove the water by using a Pasteur pipet where a 4 ml glass vial was set underneath. The inside of the flask was washed with dichloromethane/methanol (DCM/MeOH) (6:4) and transferred to the column several times. Solvent from the vials was dried by nitrogen gas but not for long then, added with a tiny amount of

n-hexane and stored in the refrigerator for separation into lipid fractions.

Column chromatography

An aliquot of the lipid extract was separated in to three fractions (Fl: alkenes; F2: alkenones and alkenoates; F3: polar lipids) by column chromatography using an emulsion of silica gel ($Si0_2$ with 5% distilled water, 5.5 mm i.d. x 45 mm long). The column was washed first with 3 ml of n-hexane. Then the **Fl** labeled vial was set underneath added with 30 µl of 0.1 g/l n-C_{24} D_{50} as an internal standard. The extract was transferred into the column by a long Pasteur pipet and the vial containing the extract was washed with 3 ml of hexane: toluene (3:1). After which: the vial beneath the column was changed with F2 added with 50 µl of 0.1 g/l nC_{36} H_{74}(internal standard) and washed the sample vial by 4 ml of toluene. Then the vial was replaced with F3 and eluted with 3 ml toluene: methanol (3:1). Fl and F2 samples were dried under nitrogen gas stream then added with 50 µl and a certain amount of n-hexane calculated based on PCV, respectively. About 50 µl of samples were transferred into the GC vials for analysis.

Gas chromatography

The conditions used for the GC analysis of the alkenones were the same as those described by Yamamoto et al. (2000), as follows: Gas chromatography was conducted using a Hewlett Packard 5890 series Il gas chromatograph with on-column injection and electron pressure control systems and a flame ionization detector (FID). Samples were dissolved in hexane. Helium was used as a carrier gas with a flow rate of 30 cm's. The column used was a Chrompack CP-Sil 5CB (length 60 m: i.d_, 0_25 mm; thickness, 0.25 µm). For the analysis of alkenone samples, the oven temperature was programmed from 70 to 310^{0}C at 20^{0}C/min and then held at 310^{0}C for 40 minutes. While for the alkene samples, the oven temperature was programmed from 70 to 130^{0}C at 20^{0}C/min, from 130 to 310^{0}C at 4^{0}C/min and then held at 310^{0}C for more than 20 minutes.

Results and Discussion

Algal growth and changes in alkenones and alkenoates Contents in batch cultures

E. huxleyi showed optimum growth at 20^{0}C and could grow even at IO^O C in both batch and continuous cultures (Figs. 1 and 3). The growth at higher temperature reached a stationary phase earlier than that at lower temperature. The wide temperature tolerance of E. hux'eyi can explain its extensive distribution in the ocean (Roth, 1994; Winter et al 1994).

The alkenone contents (pg cell-') in cells grown at 15^{0}C, 20^{0}C and 25^{0}C changed during growing phase and attained at almost similar level at the stationary phase during batch culture (Fig. 1B). On the other hand, at 10^{0}C that is a critical temperature for growth of *E. huxleyi*, alkenones were rapidly synthesized for about 6 days and continued further with a slow rate. This conspicuous increase of alkenone content at 10^{0}C is the consequence of the apparent increased production of $C_{37:3}$ alkenones even with a decrease in C alkenones (Figs. 2A-B). The ratio of content of $C_{37:3}$ to $C_{37:2}$ alkenones showed clear temperature-dependence. The extraordinary increase in the calculated alkenone content after the stationary growth phase was due

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to remarkable decrease in cell number resulting from cell death.

The alkenone contents of *E. huxleyi* were compared with Gephyrocapsa oceanica (another coccolithophorid species capable of synthesizing alkenones) grown at Same temperatures. The alkenone contents in *E. huxleyi* (0.35 ± 0.1 and 0.36 ± O. 1 at 200C and 25cc, respectively) up to the stationary phase were higher (0.21 ± 0.05 at 20 ^{0}C) and similar (0.36 ± 0.1 at 25^{0}C) than those in G. oceanica. When the values were calculated on such a basis as packed cell volume (PCV), the alkenone contents in *E. huxleyi* (0.08 ± 0.02 and 0.12 ± 0.03 at 20^{0}C and 250C, respectively) were larger than those in G. oceanica (0.05 ± 0.02 and 0.06 ± 0.01 at 20^0 C and 25^0 C, respectively) (data not shown). Microscopic observation in this study confirmed the evidence of Shiraiwa et al. (2003) for the cell volume of G. oceanica being several times greater than that of *E. huxleyi*, especially in the stationary phase. These results indicate that the quantity of alkenones synthesized Was not influenced by the cell size: as has been suggested by Conte et aL (1998). A further investigation will be necessary to determine the most effective factor for determining or affecting the alkenone content. since the variation in alkenone and alkenoate contents was very large (0.2-1.5 pg cell-i), even within one species of *E. huxleyi* when cells isolated from various geographical locations were used (Conte et al., 1995).

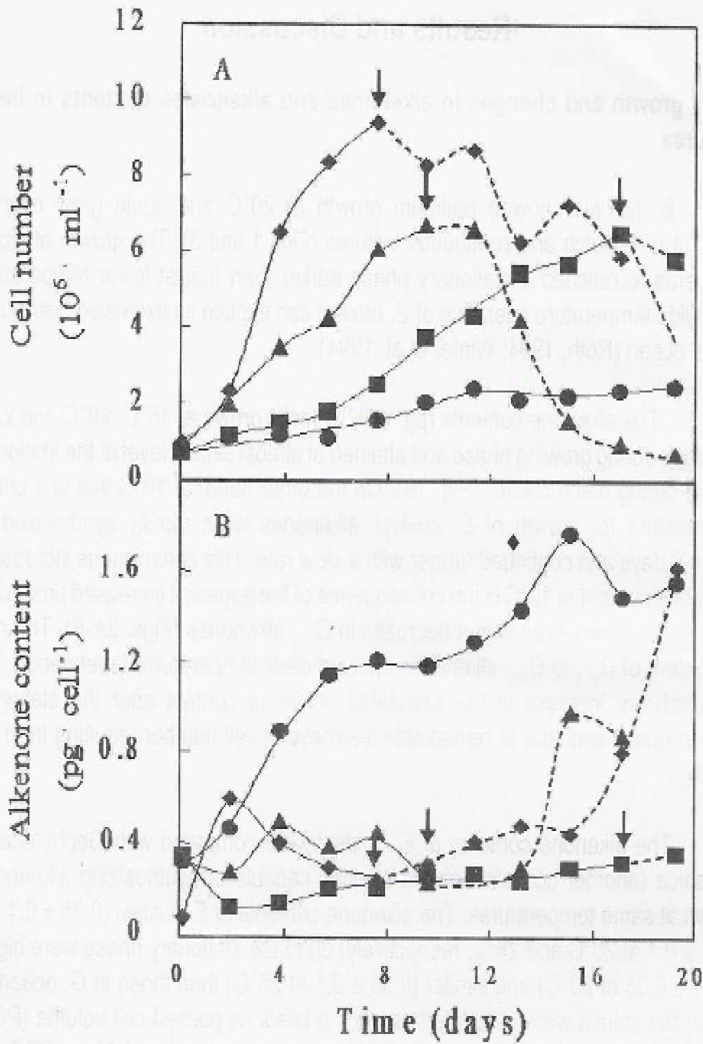

Figure 1. Changes in cell number (A) and total alkenone content (B) of *Emiliania huxleyi* grown at various temperatures including 10°C (●), 15°C (■), 20°C ( ) and 25°C (▲). Arrows indicate the time at which the growth reached stationary phase. Broken lines indicate the values after the time when cell death commenced.

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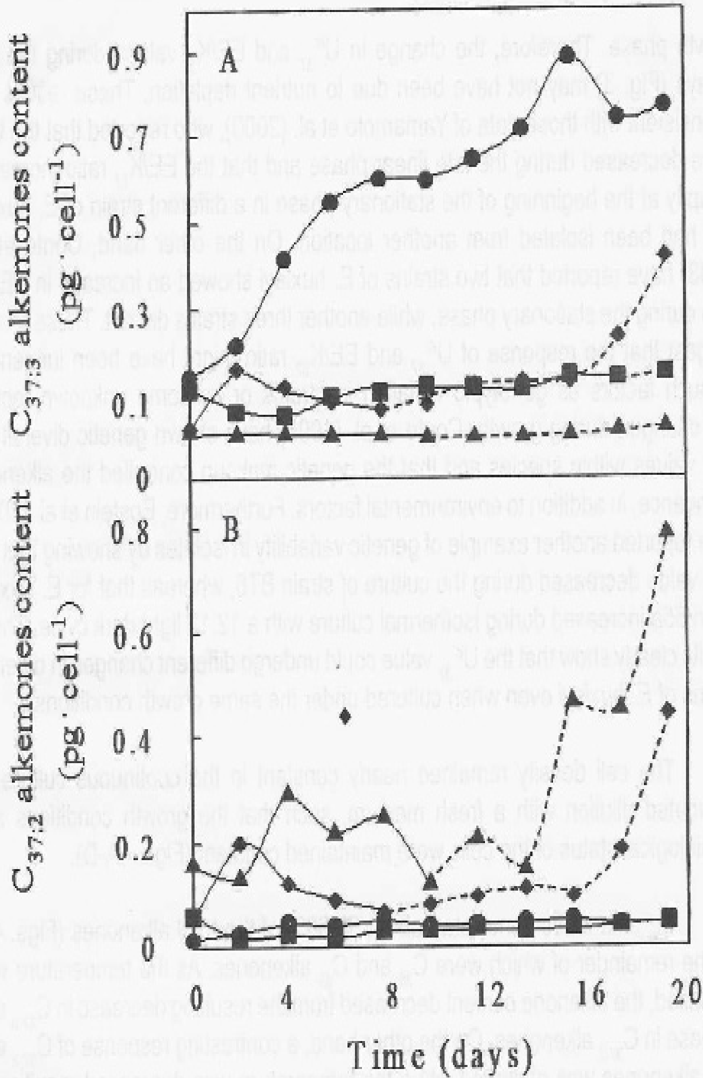

Figure 2. Changes in $C_{37:3}$ alkenone content (A) and $C_{37:2}$ alkenone content (B) of *Emiliania huxleyi* grown at 10°C (●), 15°C (■), 20°C ( ) and 25°C (▲). For arrows and broken lines, see Fig. 1.

depletion seems to have induced some physiological modification to the degree of unsaturation of alkenones in previous studies (Epstein etal,, 199b; Yamamoto et al., 2000; Versteegh 2001), and to the EE/K ratio (Yamamoto et al., 2000). However, a substantial change in the nutrient level and its depletion seem most likely to have occurred in the late linear or early stationary growth phase. Therefore, the change in and EE/K_{37} values during the first 4 days (Fig. 3) may not have been due to nutrient depletion. These results are inconsistent with those data of Yamamoto et al. (2000), who reported that the UK value decreased during the late linear phase and that the EE/K_{37} ratio increased abruptly at the beginning of the stationary phase in a different strain of *E. huxleyi* that had been isolated from another location. On the other hand, Conte et al. (1998) have reported that two strains of *E. huxleyi* showed an increase in EE/K_{37} ratio during the stationary phase, while another three strains did not. These results suggest that the response of and ratio might have been influenced by such factors as genotypic variation in strains or by some unknown factors that changed during growth. Conte et al. (1995) have shown genetic diversity in $U^{K'}_{37}$ values within species and that the genetic makeup controlled the alkenone abundance, in addition to environmental factors. Furthermore, Epstein et al. (2001) have reported another example of genetic variability in isolates by showing that the $U^{K'}_{37}$ value decreased during the culture of strain BT6, whereas that for *E. huxleyi* strain 55a increased during isothermal culture with a 12:12 light:dark cycle. These results clearly show that the $U^{K'}_{37}$ value.could undergo different changes in different strains of *E. huxleyi* even when cultured under the same growth conditions.

The cell density remained nearly constant in the continuous culture by automated dilution with a fresh medium, such that the growth conditions and physiological status of the cells were maintained constant (Figs. 4A-D).

C_{37} alkenones constituted about 50-60% of the total alkenones (Figs. 4E-H), the remainder of which were C_{38} and C_{39} alkenones. As the temperature was increased, the alkenone content decreased from the resulting decrease in $C_{37:3}$ and increase in $C_{37:2}$ alkenones. On the other hand, a contrasting response of $C_{37:3}$ and $C_{37:2}$ alkenones was observed when the temperature was decreased resulting in an increase in the alkenone content (Figs. 41-L). Our data suggest that alkenones Could be preserved in damaged cells. Rontani et a'. (1997) have reported that alkenones were not easily degraded by photochemical reactions even in broken cells of *E huxleyi*. The data from the same study also suggest that alkenones are chemically stable compounds.

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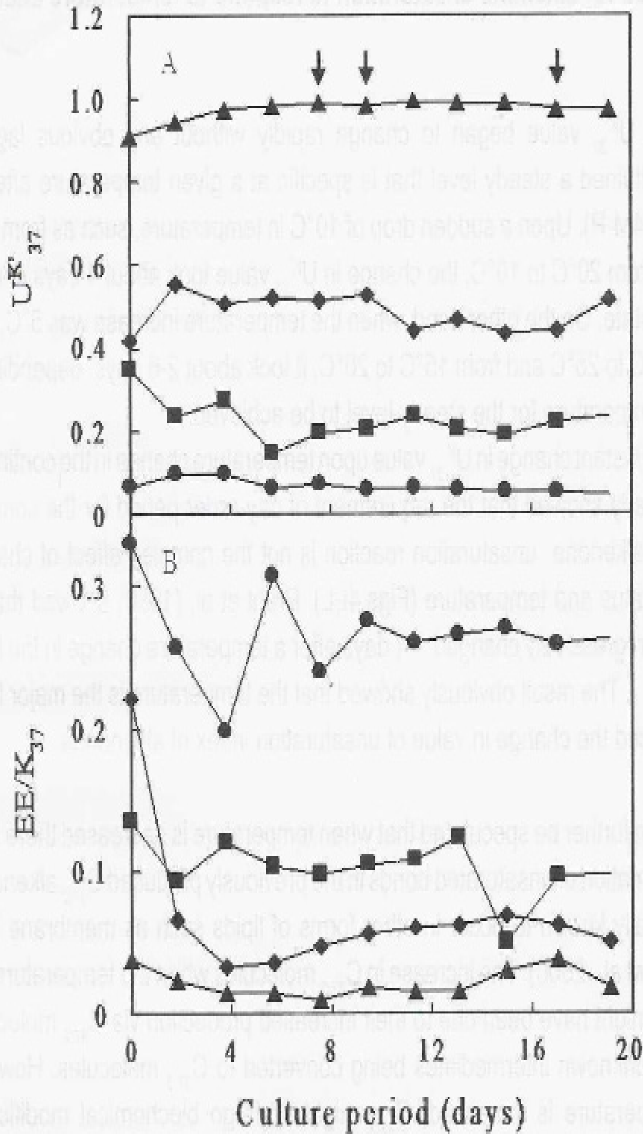

Figure 3. Changes in $U^{K'}_{37}$ (A) and EE/K_{37} (B) of *Emiliania huxleyi* (grown at 10°C (●), 15°C (■), 20°C ( ) and 25°C (▲). For arrows see Fig. 1.

(Figs. 4M-P). upon a sudden drop of 10^0C in temperature, such as from 25^0C to 15^0C or from 20^0C to 10^0C, the change in value took about 4 days to reach the steady state. On the other hand, when the temperature increase was 5^0C, such as from 20^0C to 25^0C and from 15^0C to 20^0C, it took about 2-6 days, depending on the initial temperature for the steady level to be achieved.

The instant change in $U^{K'}_{37}$ value upon temperature change in the continuous cultures clearly showed that the requirement of day-order period for the complete change of alkenone unsaturation reaction is not the complex effect of changes in growth status and temperature (Figs.41-L). Prahl et al. (1988) showed that the $U^{K'}_{37}$ value progressively changed 1-4 days after a temperature change in the batch culture of. The result obviously showed that the temperature is the major factor that Influenced the change in value of unsaturation index of alkenones.

It can further be speculated that when temperature is decreased there is the possible formation of unsaturated bonds in the previously produced $C_{37:2}$ alkenones, as is generally known to occur in other forms of lipids such as membrane lipids (Somerville et al., 2000). The increase in $C_{37:3}$ molecules when the temperature was decreased might have been due to their increased production via $C_{37:2}$ molecules, or to other unknown intermediates being converted to $C_{37:3}$ molecules. However, as the temperature is increased, $C_{37:3}$ might undergo biochemical modification into other compounds and not saturation or desaturation, because under such a condition, the degree of increase of $C_{37:3}$ was less than the degree of decrease of $C_{37:2}$, while the total amount of C_{37} alkenones decreased. The metabolic pathway for the production of C_{37} alkenones remains to be investigated.

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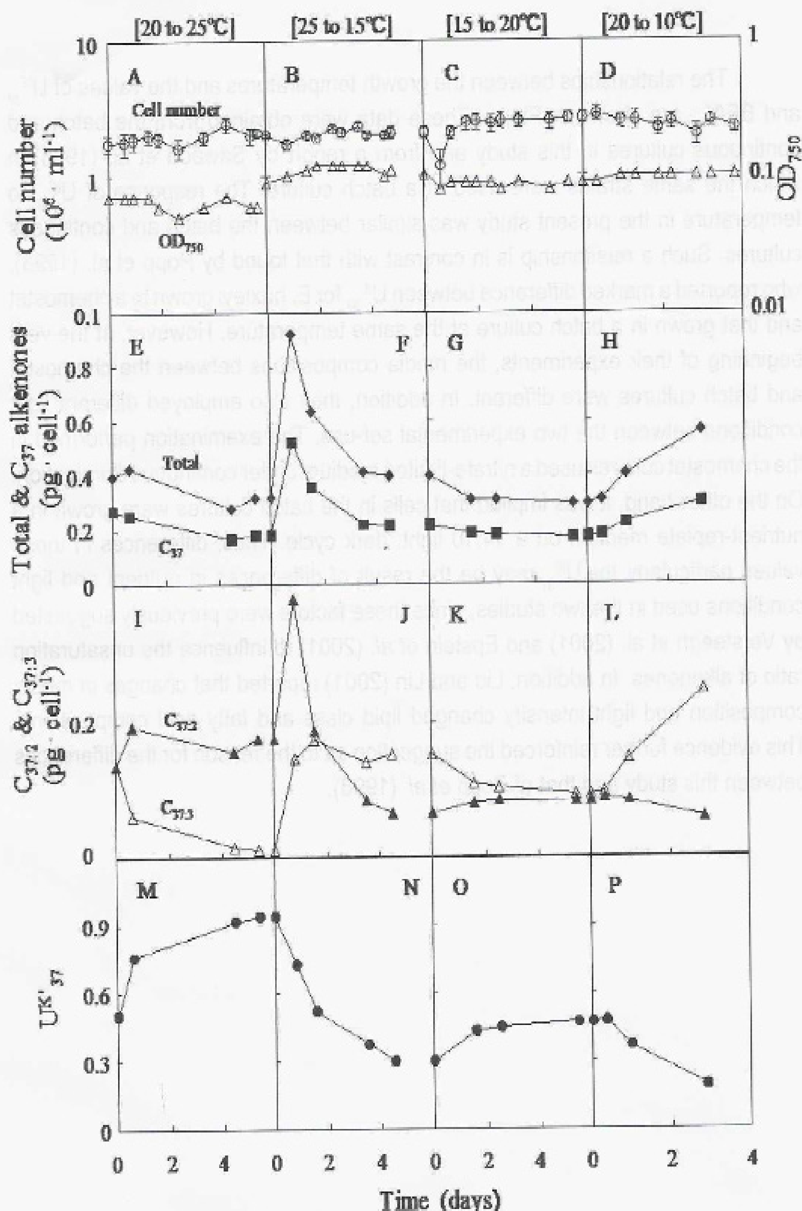

Figure 4. Changes in cell number (circles) and turbidity (triangles) (A-D); changes in the amounts (pg cell⁻¹) of total alkenones (■) and total C_{37} alkenones (■) (E-H); the amounts (pg cell⁻¹) of $C_{37:2}$ (▲) and $C_{37:3}$ (Δ) alkenones (I-L); the unsaturation index (M-P) after the transfer of cells to different temperatures in a continuous culture of *Emiliania huxleyi*.

the same strains were used in a batch culture. The response of UK'37 to temperature in the present study was similar between the batch and continuous cultures. Such a relationship is in contrast with that found by Popp et al. (1998), who reported a marked difference between UK'37 for E. huxleyi grown in a chemostat and that grown in a batch culture at the same temperature. However, at the very beginning of their experiments, the media compositions between the chemostat and batch cultures were different. In addition, they also employed different light conditions between the two experimental set-ups. The examination performed in the chemostat cultures used a nitrate-limited medium under continuous illumination. On the other hand, it was implied that cells in the batch cultures were grown in a nutrient-replete medium on a 14:10 light: dark cycle. Thus, differences in those values particularly the UK'37 may be the result of differences in nutrient and light conditions used in the two studies, since these factors were previously suggested by Versteegh et al. (2001) and Epstein et al. (2001) to influence the unsaturation ratio of alkenones. In addition, Liu and Lin (2001) reported that changes in media composition and light intensity changed lipid class and fatty acid compositions. This evidence further reinforced the suggestion as to the reason for the differences between this study and that of Popp et al. (1998).

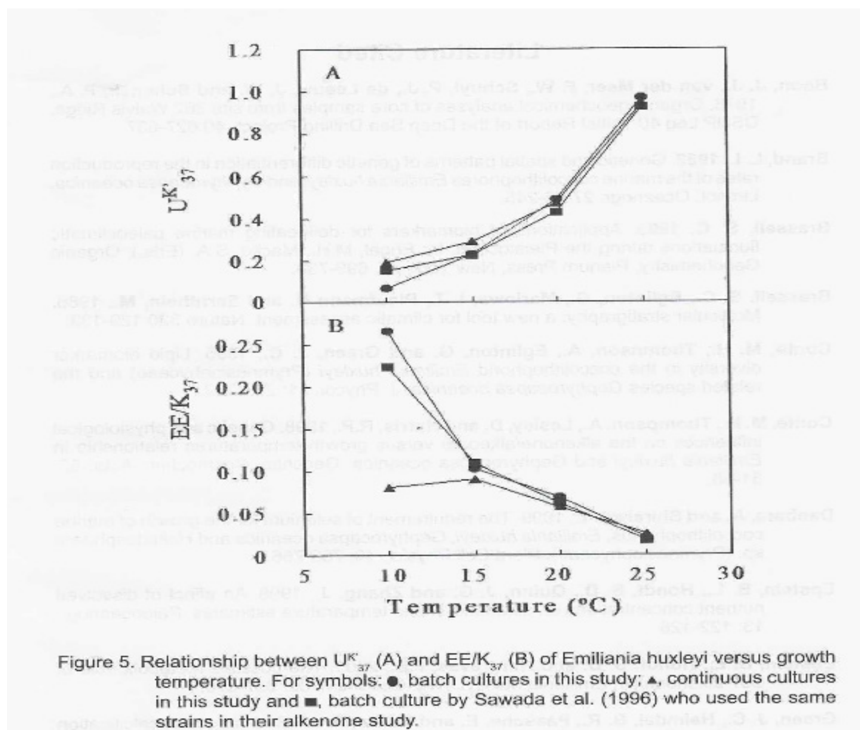

Figure 5. Relationship between U^{K}_{37} (A) and EE/K_{37} (B) of Emiliania huxleyi versus growth temperature. For symbols: ●, batch cultures in this study; ▲, continuous cultures in this study and ■, batch culture by Sawada et al. (1996) who used the same strains in their alkenone study.

Results of the present study particularly from the batch cultures were compared with the data of Sawada et al. (1996) considering that both studies used the same strain. The strain had been maintained for approximately 3 and 1 1 years under

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constant stock culture conditions until use by Sawada et al. (1996) and in this study, respectively. In E. huxleyi, the variations of UK'37 and EE/K37 among experiments were smaller than the analytical error between 15 and 250C but the difference was significant at 100C (Figs. 5A-B). Culture conditions between this study and that by Sawada et al. (1996) were similar, the only difference was the age of the strain (i.e., time after isolation). When isolated strains have been maintained under constant conditions for a long time, some physiological changes in cells might develop, leading to changes in the response of cells to environmental conditions such as alteration of physiological response to temperature change.

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